

CHITIN SYNTHESIS IN LARVAL AND PUPAL EPIDERMIS OF THE INDIAN MEAL MOTH, *PLODIA INTERPUNCTELLA* (HÜBNER), AND THE GREATER WAX MOTH, *GALLERIA MELLONELLA* (L.)

S. M. FERKOVICH, H. OBERLANDER and C. E. LEACH

Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research, Science
and Education Administration, USDA, Gainesville, FL 32604, U.S.A.

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Abstract—The level of chitin synthesis was determined in whole last-instar larvae and in pupae of *Plodia interpunctella*, and in epidermal tissue from similar stages of *Galleria mellonella*. The incorporation of radiolabelled *N*-acetyl-D-glucosamine into chitin was used to measure synthesis. Chitin production was similar in both species with peaks of synthesis occurring at the beginning of the last larval instar, in prepupae, in white pupae and prior to adult emergence. *P. interpunctella* also exhibited an additional small increase at mid-instar. Exposure of larval epidermis of *P. interpunctella* to 20-hydroxyecdysone *in vitro* stimulated chitin synthesis, but only after a 24 hr lag period subsequent to exposure to the hormone. This hormonal stimulation of chitin synthesis was inhibited by actinomycin-D and cycloheximide which suggested that 20-hydroxyecdysone-stimulated production of chitin depended on synthesis of RNA and protein. Comparison of the synthesis of chitin in epidermis of *G. mellonella* with previously published ecdysone titres, indicated that chitin production *in vivo* is preceded by an elevated ecdysone titre.

Key Word Index: All those in title plus the following: 20-hydroxyecdysone; *N*-acetyl-D-glucosamine; Actinomycin-D; cycloheximide; *in vitro*; *in vivo*.

INTRODUCTION

CHITIN is a major constituent of the insect's exoskeleton that is important in maintaining the overall structural integrity of the exoskeleton. It occurs in the form of microfibrils associated with proteins and imparts flexibility and reinforcement to the integument (HACKMAN, 1976). Biosynthesis of chitin has been studied in a number of insects (CANDY and KILBY, 1962; JAWORSKI *et al.*, 1963; SURHOLT, 1975a,b; VARDANIS, 1976; QUESADA ALLUÉ *et al.*, 1976; MAYER *et al.*, 1979). Because its synthesis is the target of a new class of insecticides (esp. diflubenzuron), considerable interest has been focused on the mode of action of inhibitors of chitin synthesis and their effectiveness in controlling insects in the field (POST and VINCENT 1973; HUNTER and VINCENT, 1974; MULLA *et al.*, 1975; TAFT and HOPKINS, 1975; SOWA and MARKS, 1975; WRIGHT and SPATES, 1976; HAJJAR and CASIDA, 1978; DUEL *et al.*, 1978; VAN ECK, 1979; MAYER *et al.*, 1980).

Chitin biosynthesis is also useful for studying the action of ecdysteroids (KIMURA, 1973; MARKS and SOWA, 1974; MARKS and SOWA, 1976; OBERLANDER *et al.*, 1978). In our laboratory we have examined the effect of 20-hydroxyecdysone on chitin production in imaginal discs *in vitro* (OBERLANDER *et al.*, 1978, 1980; FERKOVICH *et al.*, 1980). We determined in these experiments that competent imaginal discs

synthesized chitin in response to 20-hydroxyecdysone, and that this stimulation was inhibited by antibiotics known to interfere with protein or RNA synthesis. We have extended these studies to larval and pupal epidermis of the Indian meal moth, *Plodia interpunctella* (Hübner), and the greater wax moth, *Galleria mellonella* (L.). Our knowledge of chitin synthesis in imaginal discs of *P. interpunctella* made it appropriate to measure chitin synthesis in the epidermis of this species so that the production of chitin could be compared under similar conditions. Secondly, *G. mellonella* was selected for these studies so that chitin synthesis during metamorphosis could be compared with ecdysteroid levels previously determined for this insect (HSIAO and HSIAO, 1977; BOLLENBACHER *et al.*, 1978).

MATERIALS AND METHODS

Experimental insects

Plodia interpunctella was reared according to the procedure of SILHACEK and MILLER (1972). Larvae were selected for the experiments based on age and weight. The weight ranges of larvae selected from the rearing containers corresponded with the following ages (larval ages are given in days, counting from day that eggs were deposited): for 5th-instar larvae of *P. interpunctella* 3–5 mg insects were 10 and 11 days old; 6–8 mg, 11 days; 9–12 mg, 12 days; 13–16 mg, 12 days; and 17–20 mg, 12- and 13-days old; the wandering and spinning stages were 13 and 14 days old, respectively.

Mention of a commercial or proprietary product in this paper does not constitute an endorsement of that product by the USDA.

Galleria mellonella was reared according to the methods of BECK (1960). In the case of *G. mellonella* newly-moulted 7th-instar larvae were collected and used for staging subsequent larvae. Insects were selected on the basis of weight and day from moult into 7th-instar. In these experiments 60–90 mg insects were 1 day; 100–130 mg, 2 days; 140–170 mg, 2 days; 180–200 mg, 3 days; 210–240 mg, 3 and 4 days; 250–280 mg, 4, 5 and 6 days; the wandering, light spinning and spinning stages were 6- and 7-days old, respectively.

Measurement of chitin biosynthesis

Plodia interpunctella. One microlitre of *N*-acetyl-D-glucosamine, *N*-[acetyl-1- 14 C] that contained 2.5×10^{-5} mCi in ethanol: water (9:1) was injected (using a size 33 gauge needle and an ISCO micro-applicator, Model M) into the dorsal intersegmental membrane (between metathoracic legs and abdomen) of 6 groups with 5 larvae or pupae/group. The 14 C-*N*-acetyl-D-glucosamine (sp. act. 35.6 mCi/mM) was purchased from New England Nuclear, Boston, MA. The injected insects were held on cereal diet in plastic boxes (1 x 1 in.) for 20 hr. After this time they were removed from the diet and whole larvae or pupae were homogenized in 1.0 ml of 50% KOH. The homogenizer was rinsed with an additional millilitre of KOH and the samples were placed in a boiling water bath for 2 hr. The sample was then vacuum-filtered on a glass fibre filter (Gelman® A-E) that was dried and pre-weighed to collect the chitin as previously described (OBERLANDER *et al.*, 1980). After washing the filter with water and acetone, the filter plus chitin residue was dried in an oven at 100°C for 1 hr then weighed. It was then placed in 10 ml of Insta-Gel® and the radioactivity measured with a Packard (2450) Tricarb® liquid scintillation counter (79% counting efficiency).

Galleria mellonella. The procedure was the same as for *P. interpunctella*, except for the following modifications. Five microlitres (9:1 ethanol:water) that contained 1×10^{-5} mCi of isotope was injected into 3 groups of either 3 larvae or pupae per group. The large amount of fat body present in *G. mellonella* larvae and pupae interfered with filtering KOH-digested samples. Therefore, the pupae and larvae were dissected in distilled water and fat body and other organs were gently teased and washed away from the epidermis which was then homogenized in KOH. In 6-days old pupae (pharate adults), analysis of the pupal cuticle indicated an absence of radioactivity; therefore, the abdominal cuticle of the pharate adult was saved for measurement of chitin synthesis. Also, in this stage, pupae were separated according to sex and analyzed for incorporation of isotope.

To determine if the radioactivity trapped on the filters was incorporated into chitin, the following experiment was performed. The filters were incubated with and without chitinase (10 mg/ml 0.05 M acetate, pH 5.2) at 37°C for 48 hr. The chitinase was purchased from Sigma Chemical Co., St. Louis, MO. Incorporation was reduced by 40% in *P. interpunctella* whole pupae and by 87% in *G. mellonella* epidermal patches. The lower percentage inhibition obtained in the case of *P. interpunctella* may have been due to the use of whole insects which interfered with filtering the samples, thus trapping degraded chitin on the filters. In the case of *G. mellonella*, the thick, gel-like consistency of the KOH-digested whole larvae and pupae was observed to plug the membrane filter. Consequently, incorporation of the isotope was measured only in body wall. In similar experiments 94% of the radioactivity from labelled imaginal wing discs cultured *in vitro* was removed by treatment with chitinase (FERKOVICH *et al.*, 1980).

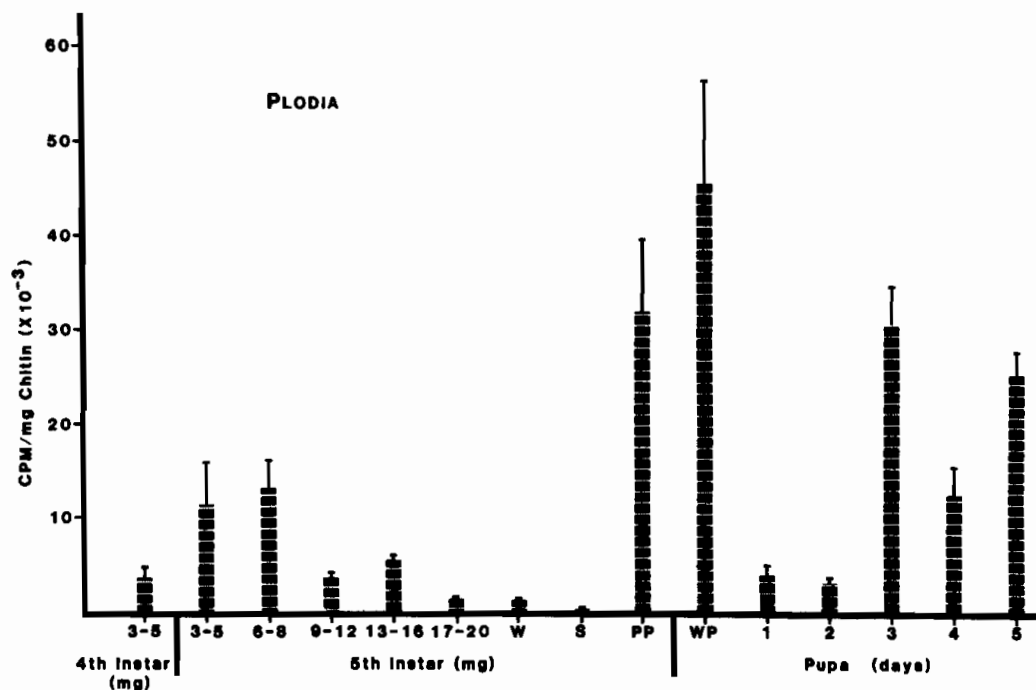


Fig. 1. The incorporation of radiolabelled *N*-acetyl-D-glucosamine into chitin of whole last-instar larvae and pupae of *P. interpunctella* in a 20 hr period after injection of the isotope. Bars represent standard error.

In Vitro methods. Epidermal patches were dissected in Grace's tissue culture medium from 18 to 21 mg last-instar *P. interpunctella* larvae. The head and terminal abdominal segments were removed, and the epidermis was cut along the mid-ventral and mid-dorsal lines. The 2 epidermal 'halves' were cleaned of adhering fat body and other tissues with watch-maker's forceps; rinsed in culture medium, and incubated for 24 hr at $25 \pm 2^\circ\text{C}$ (10 patches/culture dish) using tissue culture procedures described by OBERLANDER and LEACH (1979). After the initial 24 hr of culture, 20-hydroxyecdysone ($1 \mu\text{g/ml}$), or an equivalent volume of solvent (10% ethanol) was added to the medium. After 24 hr incubation with hormone the epidermal patches were rinsed in medium and transferred to new culture medium for 24 hr. Then, ^{14}C -N-acetyl-D-glucosamine ($2.0 \mu\text{Ci}$), (New England Nuclear; sp. act. 35.6 mCi/mmol) was added to 2 ml of the culture medium. After a 2 hr incubation with isotope the radioactivity in the chitin was measured as previously described (OBERLANDER *et al.*, 1980).

Antibiotics. Actinomycin D and cycloheximide were obtained from Calbiochem-Behring, La Jolla, CA. As a check on the activity of the antibiotics on epidermis we measured the inhibition of ^{14}C -uridine incorporation (actinomycin D) or ^3H -leucine incorporation (cycloheximide) into TCA-precipitated material. The methods used for recovery of label are described in OBERLANDER *et al.* (1980). Both isotopes were purchased from New England Nuclear and the specific activity of the ^3H -leucine was 5.0 Ci/mmol , and 464 mCi/mmol for the ^{14}C -uridine. Although these procedures do not permit conclusions about RNA and protein synthesis *per se*, they permit a comparison of the inhibition of incorporation of precursors of RNA (uridine) or protein (leucine) with the inhibition of chitin synthesis.

RESULTS

Chitin synthesis in P. interpunctella. The

incorporation of ^{14}C -N-acetyl-D-glucosamine into chitin in whole larvae and pupae of *P. interpunctella* is shown in Fig. 1. The results show peaks of chitin synthesis at the beginning of the last larval instar, in prepupae, in white pupae, and during the last days (on days 3 and 5) of pupal life before adult emergence. In addition, there was elevated chitin synthesis in the middle of the last larval instar. From these experiments we concluded that late last-instar larvae (18–20 mg) would be appropriate sources of epidermis for testing for possible ecdysteroid stimulation *in vitro* since chitin synthesis at this age was minimal.

Initially, larval epidermis from *P. interpunctella* was cultured for 24 hr either without hormone, or with 20-hydroxyecdysone and then incubated with ^{14}C -N-acetyl-D-glucosamine for 2, 4, or 6 hr. However, no significant increase in chitin synthesis occurred under these conditions. Subsequently, it was found that a 24 hr period after exposure to the hormone (72 hr of culture) was required for synthesis. Figure 2 shows that the level of incorporation of ^{14}C -N-acetyl-D-glucosamine increased linearly with time upon incubation with the isotope. After 2 hr of incubation with isotope, chitin production was $12\times$ greater relative to the control. At the 6 hr incubation time, however, incorporation of the isotope was less pronounced ($6\times$ higher).

The possible role of RNA and protein synthesis in chitin production by cultured epidermis was checked by incubating the tissue with ecdysteroid and either actinomycin-D, an inhibitor of RNA synthesis, or cycloheximide, an inhibitor of protein synthesis (see Discussion in OBERLANDER *et al.*, 1980). Relative to the control, 20-hydroxyecdysone ($1.0 \mu\text{g/ml}$) stimulated incorporation of ^{14}C -N-acetylglucosamine by 88%. Although incorporation of ^3H -leucine and ^{14}C -uridine was lower in hormone-treated epidermis relative to controls, incorporation was high enough to permit testing the effects of the protein and RNA inhibitors. Cycloheximide ($50 \mu\text{g/ml}$) and actinomycin-D ($0.5 \mu\text{g/ml}$) inhibited chitin synthesis

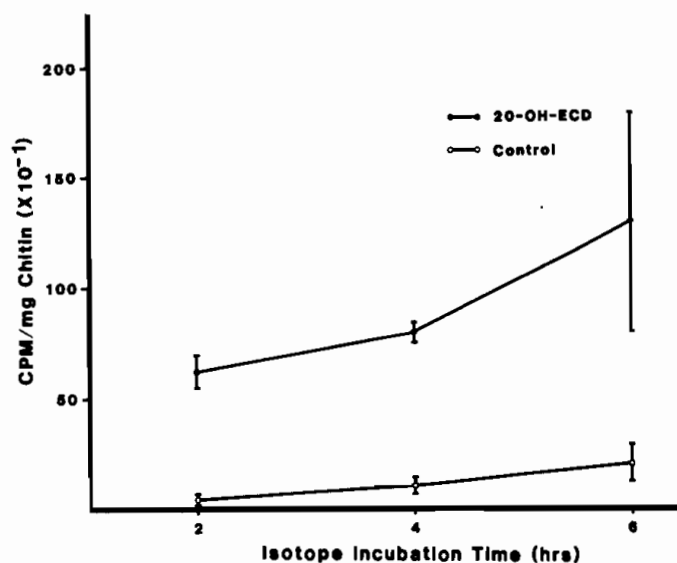


Fig. 2. The incorporation of radiolabelled N-acetyl-D-glucosamine into chitin of epidermal patches from *P. interpunctella* with increase in isotope incubation time. The tissue was held in culture medium for 24 hr, exposed to 20-hydroxyecdysone for 24 hr and held for an additional 24 hr before incubation when the isotope was measured.

Table 1. Effect of inhibitors on *P. interpunctella* epidermis *in vitro**

	¹⁴ C- <i>N</i> -Acetylglucosamine (cpm)	³ H-Leucine (cpm)	¹⁴ C-Uridine (cpm)
Untreated control	237 ± 26	8307 ± 301	10,867 ± 850
20-Hydroxyecdysone	1919 ± 333	5674 ± 78	9498 ± 1037
20-Hydroxyecdysone + 10 µg/ml cycloheximide	1795 ± 297	1602 ± 327	
20-Hydroxyecdysone + 50 µg/ml cycloheximide	281 ± 128	944 ± 64	
20-Hydroxyecdysone + 0.05 µg/ml actinomycin-D	1225 ± 57		4154 ± 367
20-Hydroxyecdysone + 0.5 µg/ml actinomycin-D	107 ± 33		2480 ± 284

* Larval epidermal patches were cultured for 24 hr prior to incubation with 20-hydroxyecdysone (1.0 µg/ml) and antibiotic for the next 24 hr (24–48 hr of culture). The tissue was incubated with ¹⁴C-*N*-acetylglucosamine at 72–74 hr of culture; or with ³H-leucine or ¹⁴C-uridine from 48 to 50 hr of culture. The data is expressed as cpm/10 epidermal patches.

by 85 and 95%, respectively (Table 1). At these concentrations the radioactivity measured in TCA-precipitable material from cultures exposed to cycloheximide and ³H-leucine or actinomycin-D and ¹⁴C-uridine was reduced by 84 and 74%, respectively, compared with cultures treated with hormone and no antibiotic (Table 1). However, at the lower concentration of cycloheximide (10 µg/ml), a 72% inhibition of ³H-leucine uptake had little effect on chitin production. Also, at the lower concentration of actinomycin-D (0.05 µg/ml) tested, ¹⁴C-uridine uptake was inhibited 46% and chitin synthesis 36% (Table 1).

Chitin synthesis in *G. mellonella*. Because of the availability of data on ecdysteroid levels in whole insects of *G. mellonella*, chitin synthesis was measured in epidermis of larvae and pupae of this species. Chitin synthesis peaked at the beginning of the last larval instar at pupation (prepupae and white pupae), and again twice just before adult emergence (days 4 and 6) (Fig. 3). In day-6 pupae, the striking increase in chitin synthesis was due to incorporation of isotope in the

pharate adult abdomen as no label was found associated with the pupal cuticle. In pupae of earlier ages where the pharate adult cuticle had not separated from the pupal cuticle, the observed incorporation was presumably due to chitin synthesized by the pharate adult cuticle that had not yet separated from the old cuticle. Additionally, in 6-day pupae there were no statistical differences (Student's *t*-test) between level of chitin production by males and females (data not shown).

DISCUSSION

The data on chitin synthesis in whole insects of *P. interpunctella* may be compared with our previous results with imaginal wing discs. There was a mid-instar peak of chitin synthesis for whole larvae, but chitin synthesis did not begin *in vivo* in imaginal discs until the prepupal stage (FERKOVICH *et al.*, 1980). This peak was also not evident in epidermal patches of *G. mellonella* and therefore is presumably due to

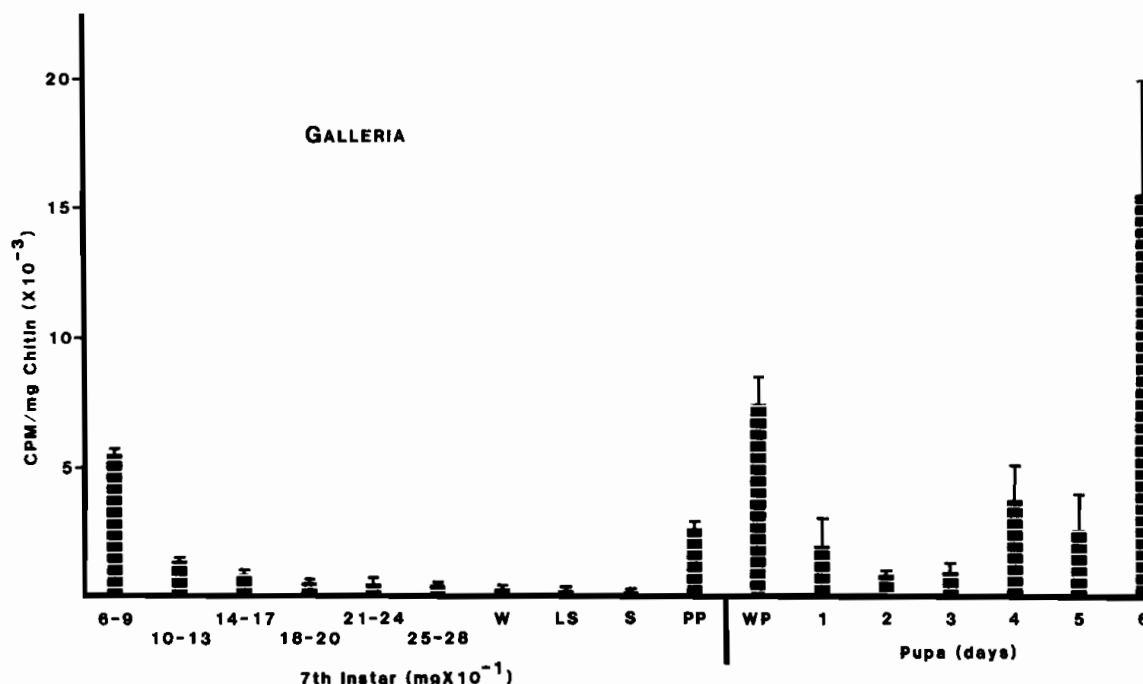


Fig. 3. The incorporation of radiolabelled *N*-acetyl-D-glucosamine into chitin of epidermal tissue of last larvae and pupae of *G. mellonella* in a 20 hr period after injection of the isotope. Day-6 pupae represents chitin synthesized by adult pharate abdominal cuticle. Bars represent standard error.

20-HYDROXYECDYSONE AND CUTICLE FORMATION

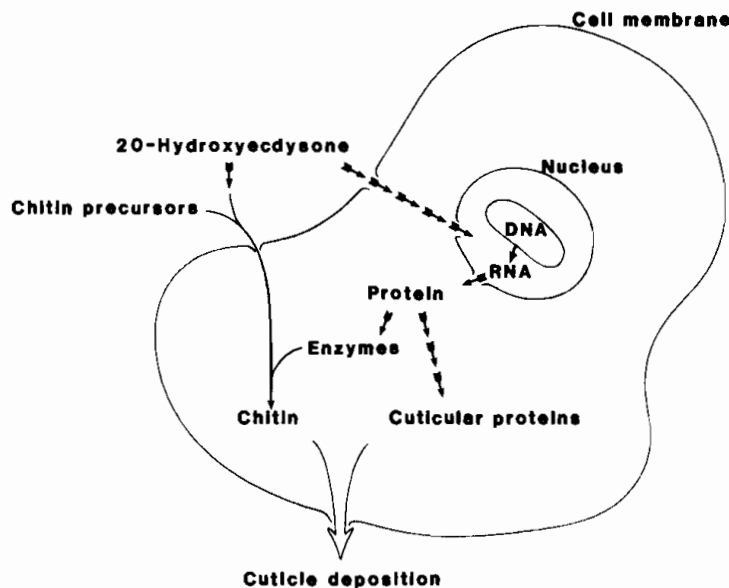


Fig. 4. Scheme for control of chitin synthesis by 20-hydroxyecdysone, based on findings with imaginal discs and larval epidermis of *P. interpunctella* and *G. mellonella*.

synthesis by some other tissues. However, the most pronounced peak of chitin synthesis in whole *P. interpunctella* which occurred in the prepupal stage coincided with the major synthetic activity for imaginal wing discs.

The pattern of chitin synthesis in larval and pupal epidermis of *G. mellonella* may be correlated with the ecdysteroid levels previously published (HSIAO and HSIAO, 1977; BOLLENBACHER *et al.*, 1978) and also the developmental events that occur with changes in the ecdysteroid titres (DEAN *et al.*, 1980). In these studies ecdysteroid titre exhibited a small peak in wandering larvae and in spinning larvae, and a major peak in day-2 pupae. Prior to emergence, the titre was high in females, but not males, the majority of this female-specific ecdysteroid remained in the ovary where it was synthesized. Our observations showed that chitin synthesis in *G. mellonella* is not coincident with ecdysteroid secretion, but increases during the period that follows a peak in the ecdysteroid level. A 1-day delay in the onset of chitin synthesis after exposure to ecdysteroid was also observed in imaginal wing discs of *P. interpunctella* *in vitro* (OBERLANDER *et al.*, 1980).

The *in vitro* experiments demonstrated significant similarities between patches of epidermis and imaginal wing discs of *P. interpunctella*. There was a lag of about 1 day between the conclusion of an adequate ecdysteroid treatment and the initiation of chitin synthesis in both tissues. Also, chitin synthesis was inhibited in both tissues by actinomycin-D and cycloheximide. Finally, the concentration and exposure period required for stimulation of chitin synthesis by 20-hydroxyecdysone in cultured epidermis was predicted on the basis of our earlier experiments with imaginal discs (OBERLANDER *et al.*, 1978; FERKOVICH *et al.*, 1980; OBERLANDER *et al.*, 1980).

Our analysis of chitin synthesis of imaginal discs of *P. interpunctella* and epidermis of *G. mellonella* *in vitro* support the concept that 20-hydroxyecdysone-

stimulated chitin production requires the synthesis of RNA and protein. MILLER and OBERLANDER (1980) have shown that new proteins (revealed by 2-dimensional electrophoresis) are synthesized by imaginal wing discs incubated with 20-hydroxyecdysone. The function of these proteins is unknown but they could include cuticle structural proteins, chitin synthetase, or activators of that enzyme.

A significant feature of ecdysteroid stimulation was uncovered in earlier experiments when it was learned that amino-sugar uptake in imaginal discs is stimulated by 20-hydroxyecdysone, and that inhibition of this uptake of precursor with cytochalasin-B blocked chitin synthesis (OBERLANDER, 1976). In this connection it should be noted that ecdysone stimulated amino-sugar uptake by imaginal discs, but did not stimulate cuticle formation. Thus, ecdysteroids may have 2 fundamental actions in the stimulation of chitin synthesis.

Our conclusions on the hormonal control of chitin synthesis in epidermis and imaginal discs are shown in Fig. 4. We propose that 20-hydroxyecdysone stimulates the uptake of amino-sugars and also the RNA-dependent synthesis of proteins, such as enzymes and structural proteins, needed for the synthesis of chitin and the formation of a complete cuticle.

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